Oncogenes and Tumor Suppressors

The HER2- and Heregulin β1 (HRG)-Inducible TNFR Superfamily Member Fn14 Promotes HRG-Driven Breast Cancer Cell Migration, Invasion, and MMP9 Expression

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Abstract

HER2 overexpression occurs in 15% to 20% of all breast cancers and is associated with increased metastatic potential and poor patient survival. Abnormal HER2 activation, either through HER2 overexpression or heregulin (HRG):HER3 binding, elicits the formation of potent HER2–HER3 heterodimers and drives breast cancer cell growth and metastasis. In a previous study, we found that fibroblast growth factor-inducible 14 (Fn14), a member of the TNF receptor superfamily, was frequently overexpressed in human HER2+ breast tumors. We report here that HER2 and Fn14 are also coexpressed in mammary tumors that develop in two different transgenic mouse models of breast cancer. In consideration of these findings, we investigated whether HER2 activation in breast cancer cells could directly induce Fn14 gene expression. We found that transient or stable transfection of MCF7 cells with a HER2 expression plasmid increased Fn14 protein levels. Also, HRG1-β1 treatment of MCF7 cells transiently induced Fn14 mRNA and protein expression. Both the HER2- and HRG1-β1–induced increase in Fn14 expression in MCF7 cells as well as basal Fn14 expression in HER2 gene-amplified AU565 cells could be reduced by HER2 kinase inhibition with laptitinib or combined HER2 and HER3 depletion using siRNA. We also report that Fn14-depleted, HER2-overexpressing MCF7 cells have reduced basal cell migration capacity and reduced HRG1-β1–stimulated cell migration, invasion, and matrix metalloproteinase (MMP)-9 expression. Together, these results indicate that Fn14 may be an important downstream regulator of HER2/HER3–driven breast cancer cell migration and invasion. Mol Cancer Res; 11(4); 393–404. ©2013 AACR.

Introduction

Breast cancer is the most common malignancy in women worldwide and in the United States approximately 40,000 women are predicted to die from this disease in 2012 (1). HER2, a member of the EGF receptor (EGFR) family, is overexpressed in 15% to 20% of invasive breast cancers and is associated with aggressive tumor behavior, decreased time to relapse, and poor clinical outcome (2). HER3, a kinase-inactive member of the EGFR family (3), is often expressed in HER2+ breast tumors (4) and it is the preferred heterodimerization partner for HER2 (5). Indeed, HER3 has been shown to play a pivotal role in mediating both HER2 oncogenesis (6) and the resistance of breast cancers to HER2-targeted therapies (7). Herigulin-1 (HRG1; also called neuregulin-1), a member of the EGF family (8), is a HER3 and HER4 ligand that elicits the formation of potent HER2–HER3 heterodimers (9). HRG1 is expressed in breast tumors (8) and has been implicated in modulating breast cancer cell invasion (10–12) and metastasis (13). Thus, the HER2–HER3 receptor pair forms a very potent mitogenic and transforming unit that promotes breast tumorigenesis. Cancer cell migration and invasion is controlled by complex signaling events that regulate cytoskeletal changes, cell–cell and cell–extracellular matrix interactions, and extracellular proteolytic activity (14). HER2/HER3 heterodimer-driven cellular migration and invasion has been previously shown to require phosphoinositide 3-kinase (PI3K) activity (15) and a variety of downstream mediators have been implicated in these cellular processes [e.g., FAK-Src (16) and matrix metalloproteinase (MMP)-9 (17)]. In regard to the MMPs, they are known to play an important role in the tumor microenvironment by mediating effects such as remodeling of the extracellular matrix, tissue invasion and intravasation, inflammation, and angiogenesis (18). HER2/HER3 signaling has been reported to upregulate MMP production and activity (17, 19, 20), but the biologic basis of this effect remains poorly understood.
TNF-like weak inducer of apoptosis (TWEAK) and fibroblast growth factor (FGF)-inducible 14 (Fn14) are a TNF superfamily ligand–receptor pair involved in many cellular processes associated with wound repair, including inflammation and angiogenesis (21, 22). TWEAK:Fn14 binding promotes Fn14 association with adaptor molecules called TNF receptor-associated factors (TRAF), which couple to various signaling pathways such as the NF-κB, mitogen-activated protein kinase (MAPK), and PI3K/Akt pathways (21). The TWEAK-Fn14 signaling axis plays an important role in regulating various aspects of tumor behavior such as growth, survival, invasion, and angiogenesis (23–29). Importantly, Fn14 is highly expressed in many solid tumors, and in some tumor types elevated Fn14 levels have been shown to correlate with disease progression and poor patient outcome (23, 25, 28). There is also evidence that Fn14 overexpression in tumor cells can activate signaling pathways and stimulate cellular processes; for example, ectopic Fn14 expression promotes glioma cell invasion by activating Rac1 and NF-κB (25).

In a previous study we showed that elevated Fn14 expression strongly correlated with the HER2+ /ER− intrinsic subtype of breast cancer and with clinical indicators of poor prognosis (26). However, the mechanisms underlying preferential Fn14 expression in HER2+ breast tumors, as well as the potential contributions of Fn14 to HER2-mediated disease, have yet to be elucidated. Here, we report that Fn14 expression is increased in mouse mammary tumor virus (MMTV)-c-Myc and MMTV-poloma middle T antigen (PyMT) transgenic mouse breast tumors with elevated Neu (HER2) levels. Also, both HER2 overexpression in MCF7 breast cancer cells and HRG1-β1 treatment of MCF7 cells induces Fn14 gene expression, and these effects are dependent on HER2/HER3 signaling. Finally, we show that stable knockdown of Fn14 in HER2-overexpressing MCF7 cells decreases basal cell migration capacity and HRG1-β1–stimulated migration, invasion, and MMP-9 expression.

Materials and Methods

Transgenic mouse models

MMTV-c-Neu mice (FVB/N-Tg(MMTV-neu)202 Mju/J; ref. 30) were purchased from Jackson Laboratories. These mice were bred and mammary tissue samples isolated as previously described (31). All MMTV-c-Neu animal studies were approved by the Case Western Reserve University (Cleveland, OH) Institutional Animal Care and Use Committee. The MMTV-PyMT mice (FVB/N-Tg (MMTV-PyVT)634 Mju/J; refs. 32, 33) were also purchased from Jackson Laboratories. Male hemizygous transgenic mice were bred to FVB/N females and at various time points wild-type and hemizygous littermates were selected, euthanized, and 5 mammary fat pad pairs were isolated, and then frozen until use. All MMTV-PyMT animal studies were approved by the University of Maryland School of Medicine (Baltimore, MD) Institutional Animal Care and Use Committee.

Cell culture and treatments

Cell lines were obtained from the following sources: MCF7, BT474, SKBR3, MDA-MB-453, AU565, and NIH3T3 (American Type Culture Collection), MCF7/HER2 (Dr. Dihua Yu, University of Texas MD Anderson Cancer Center, Houston, TX), MCF7/HER2-18 (Dr. Anne Hamburger, University of Maryland School of Medicine), NIH3T3/HER2 (Dr. Peter Choyke, NIH, Bethesda, MD), and MCF7 Ca/LTLT-Ca (Dr. Angela Brodie, University of Maryland School of Medicine). MCF7, MCF7/HER2, BT474, SKBR3, and MDA-MB-453 cells were maintained in Dulbecco's modified Eagle's medium (Cellgro) and AU565, NIH3T3, NIH3T3/HER2, and MCF7/HER2-18 cells were maintained in RPMI-1640 (Cellgro). Both cell mediums were supplemented with 10% FBS (HyClone), 2 mmol/L L-glutamine and 1% penicillin–streptomycin. MCF7/HER2 and MCF7/HER2-18 cells were additionally maintained in 750 or 500 μg/mL G418 (Cellgro), respectively. Lentivirus-infected MCF7/HER2-18 cells were additionally maintained in 0.5 μg/mL puromycin (Cellgro). Fn14 short hairpin RNA (shRNA)-448 cells expressing myc epitope-tagged Fn14 were additionally maintained in 1 μg/mL blasticidin (Sigma). MCF7 Ca and LTLT-Ca cells were grown as previously described (34). Cells were treated with the indicated concentrations of U0126, wortmannin (both from Cell Signaling Technology), lapatinib (LC Laboratories), MMP-2/MMP-9 inhibitor IV (SB-3CT; Calbiochem), MK-2206 (Alexis Corporation), EGF, HB-EGF, BTC, HRG1-α, or HRG1-β1 (all from R&D Systems).

Western blot analysis

Western blotting was carried out as previously described (35). The following primary antibodies were used: Fn14, p-HER2 (Tyr1248), p-HER3 (Tyr1289), p-Erk1/2 (Thr202/Tyr204), Erk1/2, p-Akt (S473), Akt, p-p90RSK (Ser380), p90RSK, p-p70 S6 kinase (Thr389), p70 S6 kinase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; all from Cell Signaling Technology), Neu, ErbB3, ErbB4 (all from Santa Cruz Biotechnology), EGFR, Myc, and tubulin (all from Millipore).

FACS analysis

Flow cytometry was conducted using phycoerythrin–anti-Fn14 mAb ITEM-4 and immunoglobulin G (IgG)3 isotype control (eBioscience Inc.) as previously described (26).

RNA isolation and quantitative real-time RT-PCR assays

Total cellular RNA was extracted using the RNeasy kit (Qiagen) as previously described (36). RNA was converted to cDNA using the ProtoScript AMV LongAmp Tag reverse transcription-PCR (RT-PCR) Kit (New England Biolabs) according to manufacturer's instructions. Fn14 and GAPDH mRNA levels were quantified using an ABI Prism 7900HT Real-time PCR system (Applied Biosystems) and the following primers and probes: Fn14, Cat. # Hs00171993_m1; MMP-9, Cat. # Hs00234579_m1; GAPDH, Cat. # Hs99999905_m1 (TaqMan Gene Expression Assay, Applied Biosystems).
Plasmid DNA and siRNA transfections

MCF7 cells were transiently transfected with 2 or 4 μg of the pcDNA6-HER2 expression plasmid [provided by Dr. Mien-Chie Hung (University of Texas MD Anderson Cancer Center)] per 60-mm dish using the Effectene Transfection Kit (Qiagen) according to the manufacturer’s instructions. Cells were harvested 24 hours posttransfection for Western blot analysis. In other experiments, Fn14 shRNA-448 cells were transfected with pcDNA6 vector or pcDNA6-Fn14-myc plasmid (37) as above, and drug-resistant cell colonies were selected using blasticidin. Positively transfected clones were expanded and screened for Myc levels by Western blot analysis. For siRNA experiments, MCF7, MCF7/HER2, MCF7/HER2-18, or AU565 cells were plated at a density of 2 × 10^5 in 60-mm dishes, and 24 hours later the cells were transfected with either Allstars nonsilencing control, luciferase control, HER4, HER2, and/or HER3 siRNA duplexes (Qiagen) at a concentration of 25 nmol/L using the Lipofectamine 2000 transfection reagent as previously described (26). Cells were harvested 48 hours posttransfection for Western blot analysis.

Lentiviral transduction and isolation of Fn14-depleted cell lines

Lentiviral constructs encoding control, nontarget shRNA or shRNAs targeting 2 different regions of the Fn14 transcript (Fn14 shRNA#448, Cat.# TRCN0000072448 and Fn14 shRNA#562, Cat.# TRCN0000222562) were purchased from Sigma. Lentiviral packaging was conducted as previously described (27). MCF7/HER2-18 cells were transduced with the various lentiviruses and drug-resistant cell colonies were selected using puromycin. Positively transfected clones were expanded and screened for Fn14 levels by Western blot analysis.

Scratch wound assays

Parental MCF7, MCF7/HER2-18, MCF7/HER2-18 control shRNA, and MCF7/HER2-18 Fn14 shRNA cells were plated in triplicate in 6-well cluster dishes and allowed to grow to confluence in normal RPMI growth medium (10% FBS). The confluent cell monolayers were scratched in the shape of a cross using a 200 μL pipette tip and normal RPMI growth medium (10% FBS) or low serum medium (0.5%) containing HRG1-β1 (50 or 200 ng/mL as indicated) was added to the cells. Wound closure was monitored over a 24-hour time period and photographs were taken at the 4 intersecting edges of the cross. Wound width at 0 and 24 hours was measured and the difference plotted as percentage wound closure.

Invasion assays

Parental MCF7, MCF7/HER2-18, MCF7/HER2-18 control shRNA, MCF7/HER2-18 Fn14 shRNA-562, and MCF7/HER2-18 Fn14 shRNA-448 cells (2 × 10^5), and MCF7/HER2-18 Fn14 shRNA-448 cells transfected with vector or Fn14-myc plasmid (1 × 10^5) were suspended in RPMI containing 0.5% FBS and seeded in the upper chamber of 24-well BD Butox Matrigel Invasion Chambers (BD Biosciences). Lower chambers contained RPMI with 0.5% FBS and HRG1-β1 (50 or 200 ng/mL as indicated). After 48 hours of incubation, the cells were fixed and stained as previously described (27). Cells were counted from 25 high-power fields per filter.

Gelatin zymography

MCF7/HER2-18 control shRNA cells, Fn14 shRNA-448 cells, and Fn14 shRNA-448 cells transfected with vector or Fn14-myc expression plasmid were plated in 100-mm dishes, serum-starved for 24 hours in serum-free, phenol red-free RPMI medium, and then treated with 50 ng/mL HRG1-β1. Conditioned media was collected at 0, 12, and 24 hours following treatment and concentrated using Amicon Ultra-15 Centrifugal Filter Units (Millipore). Protein concentrations were determined using the BCA protein assay (Thermo Fisher Scientific). Equal amounts of protein were subjected to gelatin zymography using Novex 10% Zymogram gels (Invitrogen) according to manufacturer’s instructions.

Results

Fn14 and HER2 are coexpressed in MMTV-c-Neu and MMTV-PyMT transgenic mouse breast tumors

Previous reports have indicated that Fn14 is highly expressed in human breast tumors (26, 38, 39); and in particular, it has been shown that Fn14 is most frequently overexpressed in the HER2+ breast cancer subtype (26, 39). To investigate this further, we analyzed Fn14 levels in the MMTV-c-Neu transgenic mouse model of Neu (HER2)-induced tumorigenesis. These mice express the rat c-Neu proto-oncogene under transcriptional control of the MMTV promoter/enhancer and develop spontaneous mammary tumors (30, 31). Fn14 expression was detected in all of the HER2+ mammary tumors examined with no expression seen in age-matched wild-type mammary glands (Fig. 1A). HER3 expression was also upregulated in the tumors, consistent with a previous report (40). We also examined Fn14 levels in the MMTV-PyMT model of breast cancer. In this model, mammary hyperplasia is detected at approximately 4 weeks of age and advanced carcinoma at approximately 12 weeks (32, 33). It has been reported that Neu (HER2) expression is activated in PyMT-driven mammary tumors, with the highest HER2 expression levels in carcinoma stage tumors (33). We detected Fn14 expression in both wild-type mammary glands and PyMT tumors isolated from 4- to 10-week-old mice, but Fn14 levels were highest in the 8- and 10-week-old HER2+ mammary tumor samples (Fig. 1B).

HER2 overexpression in human breast cancer cell lines and murine fibroblasts increases Fn14 levels

To investigate whether HER2 overexpression in breast cancer cells could directly upregulate Fn14 gene expression, we used MCF7 cells as they normally express relatively low levels of HER1 and HER2, moderate levels of Fn14 and HER4, and high levels of HER3 (Supplementary Fig. S1). We found that transient transfection of these cells with a
HER2 expression plasmid increased Fn14 protein levels (Fig. 2A). We then compared Fn14 levels in parental MCF7 cells and a HER2-overexpressing stably transfected MCF7 cell line (MCF7/HER2). HER2 overexpression also increased Fn14 levels in another stably transfected MCF7 cell line (MCF7/HER2-18; Fig. 2E). Finally, we compared Fn14 levels in MCF7 cells stably transfected with an aromatase expression plasmid versus their HER2-overexpressing letrozole-resistant counterpart (34) and in parental murine NIH3T3 fibroblasts versus a HER2-overexpressing stably transfected NIH3T3 cell line. In both cases, HER2 overexpression increased Fn14 levels (Supplementary Fig. S3).

HER2 overexpression in MCF7 cells was increasing Fn14 mRNA expression by quantitative real-time RT-PCR (qRT-PCR) analysis, and found that Fn14 mRNA levels were not significantly increased in MCF7/HER2 cells compared with MCF7 cells (Supplementary Fig. S2). HER2 overexpression also increased Fn14 levels in another stably transfected MCF7 cell line (MCF7/HER2-18; Fig. 2E). Finally, we compared Fn14 levels in MCF7 cells stably transfected with an aromatase expression plasmid versus their HER2-overexpressing letrozole-resistant counterpart (34) and in parental murine NIH3T3 fibroblasts versus a HER2-overexpressing stably transfected NIH3T3 cell line. In both cases, HER2 overexpression increased Fn14 levels (Supplementary Fig. S3).

Inhibition of HER2 kinase activity, combinatorial HER2–HER3 depletion, and inhibition of MEK1/2 and PI3K signaling downregulates Fn14 expression in HER2-overexpressing MCF7 and AU565 breast cancer cells

We tested whether HER2 tyrosine kinase activity was critical for HER2-mediated Fn14 upregulation using lapatinib, a tyrosine kinase inhibitor that targets both HER1 and HER2 (41). Lapatinib treatment of MCF7/HER2 cells inhibited HER2 and HER3 phosphorylation and significantly downregulated Fn14 expression (Fig. 3A). We also used RNA interference to deplete HER2 and HER3 expression in MCF7/HER2 cells. Cells were transiently transfected with control siRNA or HER2 and HER3 siRNA alone or in combination. Combined HER2–HER3 depletion, but not HER2 or HER3 depletion alone, markedly inhibited Fn14 expression (Fig. 3B). Lapatinib treatment and combined HER2–HER3 depletion also decreased Fn14 expression in the MCF7/HER2-18 cell line (Supplementary Fig. S4). AU565 is a breast cancer cell line with HER2 gene amplification that normally expresses high levels of HER2 and HER3 (Supplementary Fig. S1) and constitutive HER2 and HER3 tyrosine phosphorylation can be detected in these cells (Fig. 3C). Lapatinib treatment and HER2, HER3, or both HER2 and HER3 depletion significantly decreased Fn14 expression in AU565 cells (Fig. 3C and D). HER4 depletion in MCF7/HER2 or AU565 cells did not alter Fn14 expression (data not shown).

HER2 overexpression in MCF7 (Supplementary Fig. S5A and Fig. 3B) and AU565 (Fig. 3D) cells leads to constitutive activation of the MEK/ERK and PI3K/Akt signaling cascades. Therefore, we analyzed the effects of HER2 and/or HER3 depletion on p-Erk and p-Akt levels to investigate why HER2 or HER3 depletion alone decreased Fn14 levels in AU565 cells but not MCF7/HER2 or MCF7/HER2-18 cells. We found that HER2 or HER3 depletion alone had a much greater effect on p-Akt levels in the AU565 cells compared with the MCF7/HER2 cells (Fig. 3B and D). To determine the relative contribution of the MEK/ERK and PI3K/Akt pathways in regulating Fn14 expression in these 2 cell lines we treated cells with either the MAP/extracellular signal-regulated kinase (MEK)1/2 inhibitor U0126 or the PI3K inhibitor wortmannin. Each of these inhibitors reduced Fn14 expression levels in both cell lines, but Fn14
expression in AU565 cells was more sensitive to wortmannin treatment (Supplementary Fig. S5B and S5C). Treatment of AU565 cells with the Akt-specific inhibitor MK-2206 also reduced Fn14 expression levels (data not shown).

HRG1-β1 stimulation of MCF7 cells induces Fn14 gene expression

HER2 signaling in breast cancer cells can be activated by both HER2 overexpression and by ligand engagement of other EGF family members; and in particular, by HRG1 binding to HER3 (5, 42). Therefore, we next evaluated the effects of HRG1 and other EGF family ligands on Fn14 expression in MCF7 cells. Cells were treated with either the HER1 ligand EGF, the HER1/HER4 ligands heparin-binding EGF-like growth factor (HB-EGF), and betacellulin (BTC), or the HER3/HER4 ligands HRG1-α and HRG1-β1. All ligands increased Fn14 expression, but the maximal effect was seen with HRG1-β1 (Fig. 4A). The HRG1-β1 stimulatory effect was both dose-dependent (Fig. 4B) and time-dependent (Fig. 4C). Also, HRG1-β1 treatment of MCF7 cells transiently increased Fn14 mRNA levels (Supplementary Fig. S6). HRG1-β1–mediated Fn14 upregulation requires HER3 binding and/or signaling as HER3 depletion in MCF7 cells attenuated this effect (Fig. 4D). Lapatinib pretreatment of MCF7 cells also inhibited the HRG1-β1 stimulatory effect, indicating that HER2 signaling, most likely via HER2–HER3 heterodimer formation, is required for Fn14 gene induction (Fig. 4E). HRG1-β1 stimulation of AU565 cells also upregulated Fn14 expression levels (data not shown).

Fn14 depletion decreases MCF7/HER2-18 cell basal migration capacity

We compared the migratory potential of parental MCF7 cells and MCF7/HER2-18 cells using scratch wound assays. The HER2-overexpressing cells had an approximately 2.2-fold greater basal migration capacity (Fig. 5A and B). To determine the functional significance of Fn14 expression in HER2-overexpressing or HRG1-β1–stimulated breast cancer cells, we established MCF7/HER2-18 clonal cell lines stably transduced with either a control shRNA or 2 shRNAs targeting different regions of the Fn14 transcript. Effective Fn14 knockdown was confirmed by Western blot analysis (Fig. 5C). We analyzed the migratory potential of the 4 cell lines using the scratch wound assay. Fn14 depletion significantly decreased the ability of MCF7/HER2-18 cells to close the scratch wound (Fig. 5D).

MCF7/HER2-18 cells are poorly invasive in Matrigel assays using serum as the stimulus

We next tried to determine the effects of Fn14 depletion on MCF7/HER2-18 cell basal invasive capacity using modified Boyden chambers coated with basement membrane.
cells also stimulated migration and invasion, and this effect was approximately 2.9- and 4.9-fold greater, respectively, than the effect on parental MCF7 cells (Supplementary Fig. S7). Because HRG1-β1 treatment of control shRNA, but not Fn14 shRNA MCF7/HER2-18 cells, increases Fn14 expression (Fig. 6A and data not shown), we examined whether Fn14 upregulation was required for HRG1-β1–driven migration and invasion using our 4 cell lines. We found that Fn14 depletion attenuated both HRG1-β1–stimulated cell migration (Fig. 6B) and invasion (Fig. 6C). We next investigated whether reintroduction of Fn14 into Fn14-depleted MCF7/HER2-18 cells could rescue the invasion deficit. Fn14 shRNA-448 cells were stably transfected with vector control or extract (Matrigel). The MCF7/HER2-18 control shRNA cell lines did not invade through Matrigel when either 10% or 20% serum-containing medium was used in the bottom chamber as the chemottractant and the incubation period was as long as 72 hours (data not shown); consequently, we could not evaluate if Fn14 depletion in these cells would reduce basal invasion capacity.

**HRG1-β1–stimulated cell migration and invasion is attenuated in Fn14-depleted MCF7/HER2-18 cells**

HRG1-β1 treatment of MCF7 cells increases Fn14 gene expression (shown earlier) and HRG1-β1 is a potent promigratory and proinvasive factor for these cells (12). We found that HRG1-β1 treatment of MCF7/HER2-18...
a plasmid encoding myc epitope-tagged human Fn14. This plasmid did not include the Fn14 3‗-untranslated region (UTR), thus ectopic Fn14 expression should not be inhibited by Fn14 shRNA coexpression, and this was confirmed by Western blot analysis (Fig. 6D). Ectopic Fn14 expression in the Fn14-depleted MCF7/HER2-18 cells resulted in an approximately 1.8-fold increase in HRG1-β1–stimulated cell invasion (Fig. 6E).

**HRG1-β1–stimulated MMP-9 expression is attenuated in Fn14-depleted MCF7/HER2-18 cells**

It has been previously reported that HRG1-β1 treatment of MCF7 cells stimulates MMP-9 expression and activity (17), and we found that HRG1-β1–stimulated MCF7/HER2-18 cell invasion is reduced by approximately 50% when cells are treated with SB-3CT, a specific inhibitor of MMP-2/MMP-9 activity (ref. 43; Fig. 7A and B). Therefore, we determined whether Fn14 depletion had an effect on basal or HRG1-β1–stimulated MMP-9 gene expression. Real-time qRT-PCR analysis indicated that basal as well as HRG1-β1–stimulated MMP-9 mRNA expression was lower in the Fn14 shRNA-448 cells compared with control shRNA cells (Fig. 7C). Gelatin zymography assays were then conducted using conditioned media obtained from the 2 cell lines to examine MMP-9 protein levels. We found that the Fn14 shRNA-448 cells had reduced basal as well as HRG1-β1–stimulated MMP-9 gelatinolytic activity (Fig. 7D). Furthermore, forced expression of the Fn14 protein in the Fn14 shRNA-448 cells was able to increase HRG1-β1–stimulated MMP-9 expression (Fig. 7E).

**Discussion**

Overexpression of the EGFR family member HER2 occurs in 15% to 20% of all breast cancer cases and some HER2+ tumors also express the HER2-preferred binding partner HER3 (4). HRG1, a ligand for HER3, is also expressed in breast tumors (8). Both HER2 overexpression– and HRG1-mediated HER2/HER3 signaling likely contributes to breast cancer pathophysiology. In this study, we first extended our earlier findings showing a correlation between HER2 and Fn14 expression in human breast tumors (26) by showing their coexpression in mammary tumor specimens derived from MMTV-c-Neu and MMTV-PyMT transgenic mice. We then showed that (i) HER2 overexpression, achieved via plasmid transfection of MCF7 breast cancer cells or endogenous HER2 gene amplification in AU565 cells, activates HER2–HER3 signaling and increases Fn14 expression levels, (ii) HRG1-β1 treatment of MCF7 cells, which also activates HER2–HER3 signaling, can induce Fn14 gene expression, and (iii) HRG1-β1 treatment of HER2-overexpressing MCF7/HER2-18 cells further upregulates Fn14 levels, and this “super-induction” is required for maximal cell migration, invasion, and MMP-9 expression.
We identified a direct link between HER2 overexpression and elevated Fn14 levels in both MCF7 and AU565 breast cancer cells. First, we found that both transient and stable expression of HER2 in MCF7 cells increased Fn14 levels. Second, inhibition of HER2–HER3 signaling in MCF7/HER2 and AU565 cells using lapatinib significantly reduced Fn14 expression. Third, HER2 or HER3 depletion decreased Fn14 levels in AU565 cells, whereas combined HER2/HER3 depletion was required to see a similar effect in MCF7/HER2 and MCF7/HER2-18 cells. This cell line difference may be due to a more complete reduction of Akt activation in the AU565 cells following HER2 or HER3 depletion alone. This possibility is supported by our MEK, PI3K, and Akt inhibitor results, indicating that Fn14 expression in AU565 cells is predominantly driven by PI3K/Akt signaling.

HER2 overexpression can activate gene transcription via several signaling pathways, including the MAPK (44), PI3K/Akt (44), and NF-kB (45) pathways, and the Fn14 gene promotor has potential transcription factor–binding sites for both AP-1 and NF-kB (25). Therefore, we postulated that the increase in Fn14 expression in HER2-overexpressing

Figure 6. Fn14 depletion in MCF7/HER2-18 cells reduces HRG1-β1–stimulated cell migration and invasion. A, the MCF7/HER2-18 control shRNA-1A and Fn14 shRNA-448 cell lines were serum-starved overnight (0.5% FBS) and then either left untreated or treated with HRG1-β1 (50 ng/mL) for the indicated time periods. Cells were harvested and Fn14 and tubulin expression analyzed by Western blotting. B, MCF7/HER2-18 control and Fn14 shRNA cell lines were treated with HRG1-β1 (200 ng/mL) and migration capacity was compared using the scratch wound assay. Wound width was calculated at 0 and 24 hours and the difference plotted as percentage wound closure. The values shown are mean ± SEM of 3 experiments. **, P < 0.01 compared with control shRNA-1A cells by Student t test. C, MCF7/HER2-18 control and Fn14 shRNA cell lines were treated with HRG1-β1 (200 ng/mL) and invasive capacity was compared using the Matrigel invasion assay. HRG1-β1–stimulated cell invasion was quantitated as described in the Materials and Methods. The values shown are mean ± SEM of 3 experiments. **, P < 0.01 compared with control shRNA-1B cells by Student t test. D, Fn14 shRNA-448 cells, stably transfected with vector or Fn14-myc plasmid, were analyzed for Fn14-myc and tubulin expression by Western blotting. E, vector or Fn14-transfected Fn14 shRNA-448 cells were treated with HRG1-β1 (200 ng/mL) and invasive capacity was quantitated as described in the Materials and Methods. The values shown are mean ± SEM of 3 experiments. **, P < 0.01 by Student t test.
cells was likely due to an increase in Fn14 gene transcription; however, we did not observe a significant difference in Fn14 mRNA levels between the MCF7 and MCF7/HER2 cells. Thus, it appears that HER2 overexpression-mediated Fn14 upregulation is occurring via a posttranscriptional mechanism.

The HER2 receptor has no known ligand, but it can be activated following ligand engagement of other EGFR family members via receptor heterodimerization (2, 3); therefore, we examined the effects of various EGF family members on Fn14 expression in MCF7 cells. HRG1-β1, ligands for HER3 and HER4, were the 2 most potent inducers of Fn14 expression in MCF7 cells. HRG1-β1 treatment of MCF7 cells transiently increased Fn14 mRNA levels, suggesting that ligand engagement was inducing Fn14 gene transcription. Also, we found that both HER3 siRNA and lapatinib treatment prevented the HRG1-β1–stimulated increase in Fn14 expression observed in these cells, indicating that HRG1-β1–induced Fn14 expression was most likely due to HER2–HER3 heterodimerization and signaling.

We generated MCF7/HER2-18 cell lines with reduced Fn14 levels using Fn14 shRNA lentiviral constructs to investigate the potential role of Fn14 in HER2/HER3–activated breast cancer cells. First, we compared the basal migration capacity of the control and Fn14-depleted MCF7/HER2-18 cells in the presence of DMSO vehicle or SB-3CT (20 μM) in the presence of HRG1-β1 (200 ng/mL). After 48 hours of incubation, the invasive cells were stained and representative photomicrographs (×20 magnification) are shown. B, HRG1-β1–stimulated cell invasion of vehicle- or drug-treated cells was quantitated as described in the Materials and Methods. The values shown are mean ± SEM of 3 experiments.

Figure 7. Fn14 depletion in MCF7/HER2-18 cells reduces HRG1-β1–stimulated MMP-9 expression. A, MCF7/HER2-18 control shRNA-1A cells were plated into invasion chambers and treated with DMSO vehicle or MMP-2/MMP-9 Inhibitor IV (SB-3CT; 20 μM) in the presence of HRG1-β1 (200 ng/mL). After 48 hours of incubation, the invasive cells were stained and representative photomicrographs (×20 magnification) are shown. B, HRG1-β1–stimulated cell invasion of vehicle- or drug-treated cells was quantitated as described in the Materials and Methods. The values shown are mean ± SEM of 3 experiments. *P < 0.05 by Student t test. C, the MCF7/HER2-18 control shRNA-1 and Fn14 shRNA-448 cell lines were serum-starved overnight (0.5% FBS) and then either left untreated or treated with HRG1-β1 (50 ng/mL) for 12 hours. Cells were harvested, RNA was isolated, and MMP-9 and GAPDH mRNA expression was quantitated using real-time RT-PCR. MMP-9 expression was normalized to GAPDH expression. The values shown are the mean ± SEM of 3 experiments. **P < 0.05 by Student t test. D, serum-starved MCF7/HER2-18 control shRNA-1 and Fn14 shRNA-448 cells were either left untreated or treated with HRG1-β1 (50 ng/mL) for the indicated time periods. Conditioned media was collected and concentrated and MMP-2/MMP-9 activity analyzed using gelatin zymography. E, Fn14 shRNA-448 cells stably transfected with vector or Fn14-myc plasmid were serum-starved overnight (0.5% FBS) and then either left untreated or treated with HRG1-β1 (50 ng/mL) for the indicated time periods. Conditioned media was collected and concentrated and MMP-2/MMP-9 activity analyzed using gelatin zymography.
HER2-18 cell lines and found that Fn14 depletion reduced migratory capacity. This finding is consistent with previous studies showing that Fn14 depletion reduces lung (27) and prostate (28) cancer cell migration. Second, we attempted to examine the effect of Fn14 depletion on basal invasion capacity but found that the control MCF7/HER2-18 cell lines could not invade through a Matrigel barrier. Previous studies have also found that parental (26, 46) and HER2-overexpressing (47) MCF7 cells are poorly invasive. In our earlier report, we showed that ectopic Fn14 expression in MCF7 cells increases invasive capacity (26). Our finding that HER2 overexpression in MCF7 cells increases Fn14 levels but not invasive capacity indicates that this degree of endogenous Fn14 upregulation is not sufficient to increase MCF7 cell invasiveness under our experimental conditions.

Since HRG1-β1 treatment, like HER2 overexpression, can activate HER2/HER3 signaling and upregulate Fn14 gene expression in parental MCF7 cells, we investigated whether HRG1-β1 treatment of the control shRNA-expressing MCF7/HER2-18 cells might increase endogenous Fn14 levels over and beyond the level observed with HER2 overexpression alone. We found that this was the case; in addition, we showed that HRG1-β1 treatment of control MCF7/HER2-18 cells, like parental MCF7 cells (12), increased migration and invasion. These findings allowed us to test whether Fn14 upregulation was required for HRG1-β1–stimulated MCF7/HER2-18 cell migration and invasion, and this turned out to be the case. We confirmed that Fn14 levels could regulate invasion by showing that ectopic expression of Fn14 in Fn14-depleted MCF7/HER2-18 cells stimulated HRG1-β1–mediated cell invasion.

It has been reported that HRG1-β1 treatment and HER2/HER3 signaling in breast cancer cells can upregulate MMP-9 production (17) and we found that maximal HRG1-β1–stimulated MCF7/HER2-18 cell invasion requires MMP-9 activity. Since TWEAK-Fn14 binding can upregulate MMP-9 expression in various cell types, including glioma cells (48) and prostate cancer cells (28), we investigated whether Fn14 levels could also modulate MMP-9 expression. We found that in comparison with the control MCF7/HER2-18 cells, the Fn14-depleted cells had lower basal as well as HRG1-β1–stimulated MMP-9 expression. This finding is consistent with a recent report showing that transient Fn14 depletion in PC-3 prostate cancer cells can decrease basal MMP-9 expression (28).

In conclusion, we have shown that ectopic HER2 overexpression in MCF7 cells, endogenous HER2 overexpression in AU565 breast cancer cells, and HRG1-β1 stimulation of parental as well as HER2-overexpressing MCF7 cells increases Fn14 gene expression. These effects are most likely mediated via HER2/HER3 activation and stimulation of the extracellular signal–regulated kinase (ERK) and PI3K/Akt signaling pathways. We also found that HRG1-β1 treatment of HER2-overexpressing cells promotes cell migration, invasion, and MMP-9 expression, and these effects are attenuated in Fn14-depleted cells. Our finding that HRG1-β1–mediated Fn14 upregulation in MCF7/HER2-18 cells is required for maximal MMP-9 production may explain, at least in part, why HRG1-β1–stimulated invasion is impaired in the Fn14-depleted cell lines. Taken together, our results indicate that Fn14 may play a role in HER2/HER3–driven breast cancer cell migration and invasion. Furthermore, since Fn14 and HER2 are frequently coexpressed in human breast tumors (26, 39), agents targeting the Fn14 protein could be particularly beneficial to those HER2+ patients with intrinsic or acquired resistance to the current therapeutic agents trastuzumab (49) and lapatinib (50).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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